IDENTIFICATION AND CHARACTERIZATION OF AN INHIBITORY METAL ION-BINDING SITE IN FERROCHELATASE*

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Running Title: Ferrochelatase mechanism

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Ferrochelatase catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme. The severe metal ion substrate inhibition observed during in vitro studies of the purified enzyme is almost completely eliminated by mutation of an active site histidine residue (H287 – murine ferrochelatase numbering) to leucine, and reduced over two orders of magnitude by mutation of a nearby conserved phenylalanine residue (F283) to leucine. Elimination of substrate inhibition had no effect on the apparent $V_{max}$ for $\text{Ni}^{2+}$, but the apparent $K_m$ was increased one hundred-fold, indicating that the integrity of the inhibitory binding site is important for the enzyme to turn over substrates rapidly at low micromolar metal ion concentrations. The inhibitory site was observed to have a $pK_a$ value of 8.0, and this value was reduced to 7.5 by the F283L mutation, and to 7.4 in a naturally occurring positional variant observed in most bacterial ferrochelatases, murine ferrochelatase H287C. A H287N variant was also found to be substrate inhibited, but unlike the H287C variant, pH-dependence of substrate inhibition was largely eliminated. The data indicate that the inhibitory metal ion-binding site is composed of multiple residues but primarily defined by H287 and F283, and is crucial for optimal activity at low metal ion concentrations. It is proposed that this binding site may be important for ferrous iron acquisition and desolvation in vivo.

INTRODUCTION

Ferrochelatase (EC 4.99.1.1) catalyzes insertion of ferrous iron into protoporphyrin IX, and thereby represents the biochemical convergence of the coordinately regulated iron delivery and porphyrin biosynthesis pathways (1). Ferrochelatase mutations that lower overall activity below a certain threshold of about 35% result in erythropoietic protoporphyria (EPP), a painfully debilitating disease characterized by abnormally high levels of protoporphyrin IX (2). If severe enough the accumulation of this toxic biochemical intermediate in bodily tissues can lead to extreme photosensitivity and liver failure (3).

In recent years there has been some debate in the literature regarding the mechanism underlying the strict substrate specificity of ferrochelatase in vivo, where only Fe$^{2+}$, and to a far lesser extent, Zn$^{2+}$, are incorporated into protoporphyrin IX (4-6). One reason this is currently an area of interest is the remarkable metal ion substrate promiscuity of ferrochelatases in vitro, which catalyze insertion of a variety of divalent transition metal ion substrates into protoporphyrin IX with kinetic constants similar or equivalent to those for Fe$^{2+}$. While it remains unproven, it seems to be generally accepted that under in vivo conditions the oxidatively unstable ferrous iron substrate is most likely channeled directly to ferrochelatase by an undiscovered transporter or chaperone (1,6,7). The mechanism of Fe$^{2+}$ delivery and acquisition is limited by the observation that eucaryotic ferrochelatases are homodimers wherein the active site crevices are peripherally oriented into the inner mitochondrial membrane (8). This suggests the iron atom must be transported directly through the membrane, or...
the active site intermittently reorients away from the membrane and towards a matrix soluble iron chaperone. In either case this hypothetical channeling mechanism is expected to involve an iron specific transporter protein, which would explain the substrate specificity observed in vivo.

Despite these expectations, there is currently no definitive kinetic or structural evidence for direct channeling of iron to ferrochelatase. It was recently shown that human ferrochelatase can form an immuno-precipitable complex with mitoferrin (7), an inner mitochondrial membrane iron transporter (9,10), and yet the mechanism whereby a trans-membrane transporter might deliver iron to a membrane embedded active site is unclear. Nevertheless, iron channeling to ferrochelatase via mitoferrin represents a viable alternative to several other studies that have focused on interactions with the matrix soluble iron chaperone frataxin, which also interacts with mitoferrin and is essential for heme biosynthesis (11-15).

Activity profiles of ferrochelatases from yeast, mouse, and human sources have led investigators to suggest the presence of two catalytically important metal ion-binding sites in ferrochelatase (6,16). Specifically, in addition to the catalytic metal ion-binding site, an additional binding site is responsible for the substrate inhibition observable in activity profiles. In this report, we identify residues comprising the second binding site and report the beneficial role of this site in ferrochelatase activity at low micromolar metal ion concentrations. The unusual location of this binding site within a conformationally dynamic active site π-helix may offer important clues as to how iron acquisition for heme biosynthesis is realized in vivo.

**Experimental Procedures**

**Reagents** — MOPS \(^1\), Tween-80, sodium chloride, cobalt chloride hexahydrate, zinc chloride, nickel chloride hexahydrate, and cupric chloride dihydrate were from Sigma. Ferrous chloride tetrahydrate was obtained from Fisher Chemical Company. Protoporphyrin IX from Frontier Scientific. Blue Sepharose was from GE Healthcare Life Sciences. PIPBS buffer was from GFS Chemicals.

**Construction, overexpression, purification, storage, handling, and analysis of murine ferrochelatase** — The H287L and H287N variants were produced using “megaprimer” mutagenesis (17), while the H287L and F283L variants were produced and isolated from a random mutagenesis library designed and screened for directly evolved variants of ferrochelatase with elevated activity at high metal ion concentrations (data not shown). The coding regions of the variants were sequenced and the mutation encoding fragments subcloned (18) into the vector backbone. Wild-type recombinant murine ferrochelatase and the variants were overexpressed, purified, stored, and handled as previously described (18). Protein concentrations were determined spectrophotometrically using the calculated extinction coefficient of 48,500 L/(M·cm) at 280 nm based on the primary amino acid sequence encoded by the cDNA for the recombinant wild-type enzyme (19). Reported enzyme concentrations are thus in terms of monomers, or active sites.

**Buffers** — Buffers were devoid of strong metal-complexing agents such amines and sulfhydryls in order to avoid complex equilibria effects on activity measurements (16,20-23). Buffer A was defined as 0.1 M MOPS, 0.4 M sodium chloride, and 0.2% (v/v) Tween 80, pH 7.00, while buffer B, which was utilized for pH variation studies, was 20 mM MOPS, 20 mM PIPBS, 0.4 M sodium chloride, and 0.2% (v/v) Tween 80, at pH 5.75-9.25.

**Preparation of protoporphyrin IX and metal ion solutions** — The details of these procedures have been described elsewhere (16).

**Determination of steady-state kinetic parameters** — Enzyme activities were determined by monitoring the change in porphyrin absorbance, as described previously (16). In experiments where the protoporphyrin IX concentration was held constant at 20 µM, nickel-protoporphyrin formation was monitored at 562 nm and zinc-protoporphyrin formation was monitored at 584 nm, where the extinction coefficients were determined to be 20,720 L·M\(^{-1}\)·cm\(^{-1}\) and 8060 L·M\(^{-1}\)·cm\(^{-1}\), respectively. Determination of the extinction coefficients was as previously described (6,16).

Data points were fitted using SigmaPlot (Systat Software), and either the Michaelis-
Menten equation (eq. 1) or a substrate-inhibited reaction equation (eq. 2).

$$\text{rate} = \frac{V_{\text{app}}^{\text{max}} [Me^{2+}]}{K_m^{\text{app}} + [Me^{2+}]} \quad \text{(eq. 1)}$$

$$\text{rate} = \frac{V_{\text{app}}^{\text{max}} [Me^{2+}]}{K_m^{\text{app}} + [Me^{2+}] + [Me^{2+}]^2 / K_i^{\text{app}}} \quad \text{(eq. 2)}$$

In these equations $[Me^{2+}]$ corresponds to the metal ion concentration and $K_i^{\text{app}}$ represents the apparent inhibitory constant under the conditions described. Uncompetitive inhibition is assumed in equation 2, with the numerically inconsequential caveat that in the case of ferrochelatase it appears most likely that the inhibitory metal ion binds to the product-bound enzyme, as described by equation 3, rather than the Michaelis complex (6).

$$E+P \xrightleftharpoons[K_{D1}]{K_{D2}} EP + M \xrightleftharpoons{K_i}{EP} \xrightarrow{k_{\text{cat}}}{E+Pr} \quad \text{(eq. 3)}$$

Here, $E$ represents the enzyme, $P$ is protoporphyrin IX, $M$ is metal, and $Pr$ is metalloporphyrin product. The catalytic rate is defined by the rate of product release, as demonstrated in stopped-flow experiments (24).

**pH-dependence of substrate inhibition** — Apparent inhibitory constants were determined at several pH values using equation 2 and then fit to equation 3 using SigmaPlot (25),

$$K_i^{\text{app}} = \frac{1 + (K_a / [H^+])^2}{K_i (K_a / [H^+])^2} \quad \text{(eq. 3)}$$

where $K_a$ represents the dissociation constant for the inhibitory metal ion binding site, $K_i$ is the pH-independent value of the apparent $K_i$ at high pH, and $[H^+]$ is the hydronium ion concentration. This equation assumes that the inhibitory metal ion binds exclusively to a deprotonated form of the enzyme.

## RESULTS

**Activity profiles for ferrochelatase variants** — Purified ferrochelatase is subject to substrate inhibition by $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Cu^{2+}$ ions when assayed in the absence of metal ion complexing buffer components (6,16). $Zn^{2+}$, $Co^{2+}$, and $Cu^{2+}$ ions are highly inhibitory, and precise determination of kinetic and inhibitory constants is difficult, but $Ni^{2+}$ ion is less inhibitory, with an inhibition constant of $70 \pm 10 \mu M$ at pH 7.0, and this metal ion is therefore more suitable for robust characterization of the inhibitory metal ion-binding site (16). As seen in Fig. 1, the F283L mutation partially alleviated metal ion substrate inhibition by $Ni^{2+}$, while the H287L mutation eliminated inhibition altogether. Alleviation or elimination of substrate inhibition resulted in higher absolute levels of activity being observed for these variants at higher concentrations of $Ni^{2+}$, as seen in Figure 1A. However, examination of the low micromolar range, as in Figure 1B, revealed that the mutations resulted in substantially less activity at these more physiologically relevant metal ion concentrations. Fits of data at pH 8.0 indicated that the mutations not only increased $K_i$ (F283L) or eliminated inhibition (i.e., H287L, $K_i \rightarrow \infty$), but also increased the apparent $K_m$, whereas maximal velocities were not affected (Table 1). These data suggested that the primary player in the inhibitory binding site was $H287$, with the nearby $F283$ having a marked influence on binding at $H287$, and that the capacity to bind metal ion tightly at $H283/F287$ might correlate or translate into stronger binding at the catalytic site, which is defined by the $H209/E289$ pair (26).

**pH-dependence of metal ion substrate inhibition** — Metal ion substrate inhibition was pH-dependent for the wild-type, F283L, and H287C variants, partially pH-dependent for the H287N variant, and pH-independent for the H287L variant (Figures 2-4). Inhibition increased with pH for each titratable variant, consistent with tighter binding to a deprotonated inhibitory site, with the important caveat that the H287C variant was unique in that the specific activity increased at more acidic pH values, indicating this mutation converted the higher activity form of the enzyme from deprotonated to protonated. Direct comparison of activities and inhibition constants for H287C with the other variants was complicated by a lack of detectable activity with $Ni^{2+}$ as substrate, which necessitated the use of $Zn^{2+}$ to assess the kinetic characteristics of H287C. The H287N and H287C mutations both resulted in
substantial changes in the activity profiles, but these naturally occurring positional variants, like the wild-type enzyme, were strongly substrate inhibited. The weaker binding at the secondary site in the H287L and F283L variants can be viewed in Figure 2, where the metal-ion concentrations necessary to complete the activity profiles were increased twenty- and ten-fold, respectively.

The pH analyses were extended in order to ascertain the pKa for the inhibitory binding site, as depicted in Figure 3. Activity profiles were generated across a range of pH values and the apparent pKi values determined at each pH to construct a log-log plot of pKi values versus pH. Substrate (Ni2+) inhibition of the wild-type enzyme increased with pH and then plateaued above pH 8.0 at less than 10^{-5} M. The inflection point defining the pK_a for inhibition was estimated to be 8.0. The F283L variant was similar in shape but the pK_a was lowered by about 0.5 pH units and inhibition plateaued at only 10^{-4} M, more than an order of magnitude lower than the unaltered enzyme.

In contrast to the wild-type ferrochelatase and F283L variant, the inhibitory constant of the H287N variant was only mildly pH-dependent and no inflection point was observed over the range in which the enzyme was active. This result indicated the H287N mutation largely eliminated the titratable nature of the inhibitory binding site. H287L was not substrate inhibited by Ni^{2+} at any pH tested, but was inhibited by Co^{2+}, Zn^{2+}, and Cu^{2+} (See Table 2 and Figure 5). The relative errors in the determined inhibitory constants increased substantially at higher K_i values, but clear differences were observed.

The pH-dependence of inhibition for the H287C variant with Zn^{2+} as substrate was similar to the wild-type and F283L variant with Ni^{2+} (Figure 4.), with the estimated pK_a being lowered by about 0.6 pH units as a result of the mutation. The H287C mutation resulted in an enzyme that was more active than wild-type ferrochelatase at lower pH values, consistent with cysteine being found at this position in most bacteria, but this was not investigated further.

A metal ion-binding side chain at the position of H287 is not absolutely necessary for substrate inhibition to be observed — As noted above, although the H287L variant was not inhibited at any pH by Ni^{2+}, it was observed to be weakly inhibited by Co^{2+} and Zn^{2+}. This can be observed in Figure 5, where at pH 7.0 an inhibitory constant of 250 ± 30 µM was observed. This result suggests that the inhibitory metal ion-binding site is comprised of one or more other residues, in addition to H287.

**DISCUSSION**

The in vivo substrate of ferrochelatase is Fe^{2+} (1). Ferric iron (Fe^{3+}) is not a substrate, and a key outstanding question in the biochemistry of ferrochelatase is the mechanism whereby the oxidatively unstable ferrous iron substrate is acquired in vivo. The orientation of the enzyme active site into the periphery of the inner mitochondrial membrane (8) suggests a membrane transporter such as mitoferrin might deliver ferrous iron to ferrochelatase, while other studies have implicated the matrix soluble iron chaperone frataxin in interacting with ferrochelatase with iron delivery for heme biosynthesis (11-15).

We and others previously reported that ferrochelatase is subject to metal ion substrate inhibition during in vitro assays when complexing agents are eliminated from the assay buffer, which facilitated the discovery of the existence of a second catalytically important metal ion-binding site on the enzyme (6,16). In this report, we identify murine ferrochelatase active site residues F283 and H287 as key components of the second binding site observed during in vitro assays, and demonstrate that this “inhibitory site” may not be inhibitory at all in vivo, as it actually enhances activity at low µM metal ion concentrations.

A compelling argument can be made that this second ferrochelatase metal ion-binding site is conserved in nature, and possibly even fine-tuned to support the unique ecological niche of the host organism. F283 is one of only six ferrochelatase amino acid residues known to be perfectly conserved (16), and resides at the bottom of the active site cleft at the end of a conformationally dynamic π-helix (5,27). The absolute biological requirement of a phenylalanine residue at this position is somewhat perplexing, since several other amino acids have similar chemical properties, and the results presented here indicate that F283 is not essential for high levels of activity.
in vitro, and yet no replacements have been observed in nature. Four amino acids towards the carboxy-terminus from this residue is a histidine that is also conspicuously located at the back of the active site, about ten angstroms away from the invariant histidine-glutamate pair reported to form the catalytic insertion site (26). Cadmium soaked crystals of B. subtilis ferrochelatase were found to have metal ion bound at both of the equivalent histidines simultaneously, clearly demonstrating the metal binding capacity of the secondary site proposed here (28). Murine ferrochelatase H287 is conserved in eukaryotes (with the exception of the parasitic Plasmodium and Toxoplasma, which have a cysteine at this position; see Table 3) and many bacteria, but is replaced by a cysteine in most prokaryotes, and by an asparagine in a small number of infectious bacteria including Rickettsia, Campylobacter, and Helicobacter, as well as symbiotic nitrogen fixing cyanobacteria. No other amino acids are observed at this position, and this in itself is intriguing from an evolutionary perspective because interconversion of either histidine or asparagine to cysteine requires at least two point mutations, and no single point mutation intermediates are currently observed in nature, suggesting an unlikely double mutational event, or a now extinct primordial intermediate. In either case there would appear to be an unusually strong selective pressure towards these three particular metal ion-binding amino acids within the organisms that harbor them. The unusual natural distribution makes it tempting to speculate that the presence of cysteine or asparagine at this position confers some infectious capacity to many organisms, perhaps via iron related oxidative toxicity or higher activity in more acidic pH environments, but direct experiments have not yet been conducted to test these possibilities. The murine ferrochelatase H287C mutation increases activity at lower pH values, which suggests a clear selective advantage to placing cysteine in this position in anaerobic organisms, while binding at the secondary site in the H287N variant is almost pH-independent, which allows it to remain a functional metal ion binding site at lower pH as well.

It had been posited that the secondary binding site might function to confer some specificity for iron over other metal ion substrates (16), but this is not supported by the work presented here, and it now appears more likely that the additional binding site functions to strengthen overall metal binding affinity and thereby enhance activity. This is more consistent with another functional role we had postulated, which was that the second metal ion binding site is part of a substrate processing pathway that also enhances overall binding (16). The spatial positioning of H287 within a π-helix that unwinds during product release may offer important clues as to the true significance of this secondary binding site. As delineated in Figure 6, the function of the second metal ion-binding site in vivo could be to assist in transport of Fe^{2+} by E289 into the catalytic insertion site, in a way that not only precludes the toxicity that would be associated with release and oxidation, but also promotes desolvation as a means to enhance reactivity with protoporphyrin IX (please view the video supplied as Supplemental Data). In this model, which is based on interpolations of known crystal structures, F283 “sweeps” around H287 and the Fe^{2+} atom while it is within binding distance of both E289 and H287, immediately prior to its arrival at the catalytic site. In this way F283 might promote reactivity via desolvation of the Fe^{2+} atom, or modulate the interaction of the metal ion with H287 such that it is tightly bound initially, but then rapidly released from H287 into the catalytic site upon reformation of the π-helix. It is even possible that the H287/F283 binding site acts as part of a molecular switch that delivers iron to the active site. For instance, it is possible that binding of protoporphyrin IX, which is believed to involve direct channeling from protoporphyrinogen oxidase in the inner mitochondrial membrane, triggers unwinding of the π-helix, creating a transitory matrix-oriented binding site for an iron chaperone, which then channels iron to the active site via a pathway that involves E289 and the H287/F283 binding site. This mechanistic model would require expansion and refinement of equation 3 to reflect the conformational status of the enzyme, particularly about the π-helix region, as described by equation 4.

\[
E + P \overset{K_d1}{\underset{k_{D2}}{\rightleftharpoons}} E_oP \overset{K_{insert}}{\rightarrow} E_{Pr} M \\overset{K_{conf}}{\rightarrow} E_{Pr} \overset{k_{cat}}{\rightarrow} E_{Pr} M + M
\]

(eq. 4)
Here, it is the open conformation of the protoporphyrin IX-bound enzyme (EoP; unwound π-helix) that binds the iron atom at E289, stimulating reversion to the closed Michaelis complex conformation (EPM). H287 transiently complexes the metal ion during this structural transition, and F283 enhances the binding affinity and possibly assists in metal ion desolvation. Following catalysis the product-bound enzyme (EPr) reverts to the open conformation (EoPr), as supported by recent crystallographic data (5), and the metalloporphyrin is slowly released. However, at the higher metal ion concentrations utilized during in vitro experiments with the purified enzyme, a second metal ion binds to the product-bound enzyme prior to product release, resulting in a conformational complex (ExPrM) wherein product release is inhibited. This admittedly speculative but testable catalytic model suggests the inhibited complex should be found to resemble that shown in Figure 7, where the metal ion is bound by H287 and the catalytic residues, with F283 stabilizing the complex. Ironically, the substrate inhibition observed during in vitro enzyme assays may not be operable in vivo, where a channeling mechanism would ensure only one iron atom enters the active site each catalytic cycle, but could serendipitously tell us something important about iron acquisition and processing in vivo.

The H287L mutant is subject to some degree of substrate inhibition, leading us to further suggest that other residues may also be coordinately involved in metal ion-binding at the secondary site. The identity of any other binding residues is somewhat speculative, but the nearest conserved residue capable of complexes a metal ion is E293 in the π-helix, the function of which is currently unknown.

In summary, based on the data presented here we propose that metal ion substrate inhibition is an in vitro phenomenon only observable with unnaturally high substrate concentrations, and in vivo the second binding site is part of an evolutionarily conserved multi-residue substrate delivery system that transports Fe²⁺ to the active site while simultaneously preventing oxidation and promoting desolvation. The orientation of the catalytic site residue E289 into the matrix in the unwound π-helix conformation further identifies a specific and novel docking site for an iron chaperone, and implies the sought after iron transport protein should be found in the mitochondrial matrix rather than the membrane.
FOOTNOTES

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1The abbreviations used are: MOPS: 3-[N-morpholino]propanesulfonic acid; PIPBS: piperazine-N,N′-bis(4-butanesulfonic acid).

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REFERENCES

**FIGURE LEGENDS**

**FIGURE 1.** Activity profiles of murine ferrochelatase variants with Ni\(^{2+}\) as substrate. The data points correspond to ● – murine ferrochelatase; ▲ – F283L murine ferrochelatase variant; and ■ – H287L murine ferrochelatase variant. Each enzyme was at 0.2 µM, while protoporphyrin IX was held constant at 3.0 µM. The lines represent the best fit to equation 2 (wild-type and F283L ferrochelatase) or equation 1 (H287L). Specific activity is in units of micromoles metalloporphyrin produced per minute per micromole enzyme.

**FIGURE 2.** Metal ion inhibition is pH-dependent. Activity profiles at pH 7.0 (●) and 9.0 (■) were determined for murine ferrochelatase variants. Each enzyme was at 0.2 µM, while protoporphyrin IX concentration was held constant at 20.0 µM and Ni\(^{2+}\) concentration was varied. A: wild-type murine ferrochelatase; B: H287L variant; C: H287N variant; D: F283L variant; and E: H287C variant. The H287C variant was assayed with Zn\(^{2+}\) as substrate due to the absence of detectable activity with Ni\(^{2+}\) (not shown). Specific activity is in units of micromoles metalloporphyrin produced per minute per micromole enzyme.

**FIGURE 3.** Determination of the pK\(_a\) for the inhibitory binding site. Log-log plots of the inhibitory constant as a function of pH for murine ferrochelatase (●, pK\(_a\) = 7.8, K\(_i\) = 6 µM); the F283L ferrochelatase variant (▲, pK\(_a\) 7.1, K\(_i\) = 90 µM), and the H287N ferrochelatase variant (◊). In each case the enzyme was at 0.2 µM, while protoporphyrin IX concentration was held constant at 3.0 µM and Ni\(^{2+}\) concentration was varied. Reactions were conducted in buffer B at the indicated pH values.

**FIGURE 4.** Estimation of the pK\(_a\) for the H287C variant with Zn\(^{2+}\). Reactions were conducted in buffer B at the indicated pH values. The fitted pK\(_a\) value was 6.8 and the pH-independent K\(_i\) was 2 µM.

**FIGURE 5.** H287L murine ferrochelatase is substrate inhibited by Co\(^{2+}\). The reaction conditions were 0.2 µM H287L murine ferrochelatase, 3 µM protoporphyrin IX, and varying concentrations of Co\(^{2+}\) in Buffer A. Specific activity is in units of micromoles metalloporphyrin produced per minute per micromole enzyme.

**FIGURE 6.** A model for ferrochelatase iron acquisition in vivo. The protein structures in panels A and D represent the known human ferrochelatase monomeric structures in the unwound (PDB #2QD2) and wound (PDB #2HRE) π-helix conformations, respectively. Protein structures in Panels B and C are interpolations between the two conformers, as calculated by the Yale Morph Server (a movie is available as supplemental material and the interpolated structures are online at http://www.molmovdb.org/cgi-bin/morph.cgi?ID=251300-7229). The static protoporphyrin IX (green) is from PDB #2HRE, and shown for perspective of the active site, while the secondary metal ion-binding site residues corresponding to murine ferrochelatase F283 and H287 are depicted with pink carbons and the catalytic site residues corresponding to H209 and E289 are shown with yellow carbons. The position of the Fe\(^{2+}\) atom (red ball) is purely hypothetical and predicted to be defined by the position of the E289 side chain. The perspective is such that the right edge of the structures would be embedded in the matrix membrane while the remainder would be in the matrix. In A, the unwound π-helix structure, E289 points out of the back of the enzyme away from the membrane and into the mitochondrial matrix where it is positioned to accept an Fe\(^{2+}\) atom from a transporter or chaperone. In panel B the π-helix begins to reform, transporting the Fe\(^{2+}\) atom towards the active site where it encounters and binds to H287 in close proximity to F283. In panels C-D F283 is predicted to “sweep” around the bound Fe\(^{2+}\) atom as it begins to move into the active site, possibly functioning to increase reactivity by desolvating the metal atom immediately prior to release into the catalytic binding site, or favorably affecting proton transfers conducive to catalysis. This model is attractive in that it explains how a water soluble metal ion could access an active site cleft that is embedded in the highly hydrophobic mitochondrial inner membrane. It also defines a novel putative binding site for a matrix iron transport protein.
FIGURE 7. Working model for the substrate inhibited ferrochelatase complex. F283 and H287 stabilize the complex, in which product release is either prevented or greatly diminished.
## TABLE 1

Steady-state kinetic values for murine ferrochelatase variants at pH 8.0

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{app}}^{\text{max}}$ (min$^{-1}$)</th>
<th>$K_m^{\text{app}}$ (µM)</th>
<th>$K_i^{\text{app}}$ (µM)</th>
<th>$V_{\text{app}}^{\text{max}} / K_m^{\text{app}}$ (µM$^{-1}$min$^{-1}$)</th>
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<tbody>
<tr>
<td>Wild-type ferrochelatase</td>
<td>53 ± 11</td>
<td>1.0 ± 0.4</td>
<td>7 ± 3</td>
<td>53 ± 21</td>
</tr>
<tr>
<td>F283L</td>
<td>50 ± 3</td>
<td>5 ± 1</td>
<td>2400 ± 800</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>H287L</td>
<td>69 ± 1</td>
<td>116 ± 4</td>
<td>n/a</td>
<td>0.59 ± 0.02</td>
</tr>
</tbody>
</table>

## TABLE 2

Inhibitory constants for ferrochelatase variants with several divalent metal ions.$^{1}$

<table>
<thead>
<tr>
<th>FC variant</th>
<th>Fe$^{2+}$</th>
<th>Zn$^{2+}$</th>
<th>Co$^{2+}$</th>
<th>Ni$^{2+}$</th>
<th>Cu$^{2+}$</th>
</tr>
</thead>
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<tr>
<td>Wild-type</td>
<td>N.O.</td>
<td>12 ± 2</td>
<td>22 ± 3</td>
<td>70 ± 10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>H287L</td>
<td>N.O.</td>
<td>100 ± 80</td>
<td>250 ± 30</td>
<td>N.O.</td>
<td>&lt;1</td>
</tr>
<tr>
<td>H287C</td>
<td>N.O.</td>
<td>12 ± 3</td>
<td>N.O.</td>
<td>N.D.</td>
<td>&lt;1</td>
</tr>
<tr>
<td>H287N</td>
<td>500 ± 200</td>
<td>300 ± 100</td>
<td>20 ± 10</td>
<td>100 ± 30</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>F283L</td>
<td>500 ± 300</td>
<td>26 ± 7</td>
<td>44 ± 17</td>
<td>300 ± 100</td>
<td>Inconclusive</td>
</tr>
</tbody>
</table>

$^{1}$All units are in µM. N.O. stands for substrate inhibition Not Observed, and N.D. stands for activity Not Detected with this metal ion.

## TABLE 3

Evolutionary examination of the position occupied by murine ferrochelatase histidine-287

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Notable species</th>
<th>Ecological niche</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucaryotic consensus sequence</td>
<td>F-X-X-X-<strong>H</strong>-X-E</td>
<td>-</td>
</tr>
<tr>
<td>Exceptions in eucaryotic sequences</td>
<td>F-X-X-X-<strong>C</strong>-X-E</td>
<td>Plasmodium, Toxoplasma</td>
</tr>
<tr>
<td>Prokaryotic consensus sequence</td>
<td>F-X-X-X-<strong>C/H</strong>-X-E</td>
<td>-</td>
</tr>
<tr>
<td>Notable divergences from eucaryotic sequences</td>
<td>F-X-X-X-<strong>C</strong>-X-E</td>
<td>Vibrio, Yersinia, Salmonella, Haemophilus, Burkholderia, Shigella, Bordetella</td>
</tr>
<tr>
<td>All others</td>
<td>F-X-X-X-<strong>N</strong>-X-E</td>
<td>Helicobacter, Rickettsia, and Campylobacter</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Identification and characterization of an inhibitory metal ion-binding site in ferrochelatase
Gregory A. Hunter and Gloria C. Ferreira

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